**Tutorial 2 : HPLC Simulations**

In this tutorial, we use an HPLC simulator developed by the Group of analytical sciences, School of pharmaceutical sciences, University of Geneva. Download the simulator, (**Practical HPLC simulator v1.0**) in the form of an Excel file, from the address:

<https://ispso.unige.ch/labs/fanal/practical_hplc_simulator>

1. Analysis of a mixture of parabens (mix. 1)

**1.1 Physicochemical properties of the compounds**

   

Methylparaben Ethyl paraben Propylparaben Butylparaben

logP=1,67 logP=2,03 log P=2,55 logP=3,00

pKa=8,5 pKa=8,5 pKa=8,5 pKa=8,5

**1.2. Elution order.**

Consider the following operating conditions:

Column: Waters Acquity BEH C18 (150x4,6 mm, 5m).

Eluent : Acetonitrile /phosphate buffer pH=7: 40/60 v/v

Flow rate : 1mL/min

Temperature : 30°C

Detector: 220nm

 Predict the order of elution:

First eluted: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Last eluted \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Open HPLC simulator. Introduce the above operating conditions to study mixture 1. Compare the obtained order of elution with the predicted one and explain the differences.

**1.3. Dead time**

Dead time can be calculated (see tutorial 1) or evaluated by injection of unretained compounds. In this simulation, dead time is evaluated using uracil.

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logP=-0,86, pKa=8,8

Explain why this compound is unretained in these chromatographic conditions.

**1.4. Simulations**

*1.4.1. Wavelength choice*

Optimize the UV wavelength, using the spectra window and the detector window.

*1.4.2. Mobile phase:*

Modify the mobile phase composition:

-Solvent strength (% organic solvent; polarity)

-Solvent type (comparison of acetonitrile and methanol strength and selectivity)

-pH

Describe the impact on:

1. Dead time
2. Retention time
3. Retention factor (LSS model)
4. Separation (resolution)
5. Back pressure

*1.3.3. Flow rate*

Introduce the follow values of flow rate: 0,5mL/min, 1mL/min, 1,5mL/min, 2mL/min. Describe the impact on:

1. Dead time
2. Retention time
3. Retention factor
4. Separation (critical resolution)
5. Back pressure (plot: pressure = f (flow rate)

*1.3.4. Column*

-column length

-particle size

-phase type

Describe the impact on:

1. Dead time
2. Retention time
3. Retention factor
4. Separation (resolution)
5. Back pressure (plot: pressure = f(L))
6. Column efficiency (N)
   1. **Optimisation**

Choose the conditions to obtain:

- 1<k<10 and

- R>1,5

- time<15min.

2. Analysis of a mixture of acids (NAIS Mix.2)

**2.1 Physicochemical properties of the compounds**

  

Acetylsalicylic acid paracetamol salicylic acid ketoprofen

logP=1,24 logP=0,91 logP=1,98 logP=3,61

pKa=3,41 pKa=9,46 pKa=2,79 pKa=3,88

 

Ibuprofen mefenamic acid

logP=3,84 logP=5,4

pKa=4,85 pka=3,89

**2.2. Elution order.**

 Predict the order of elution:

First eluted: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Last eluted \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Introduce the following operating conditions to the simulator and study the mixture 2:

Column: Waters Acquity BEH C18 (150x4,6 mm, 5m).

Eluent : Acetonitrile /phosphate buffer pH=7: 30/70 v/v

Flow rate : 1mL/min

Temperature : 30°C

Detector: 220nm

Compare the obtained order of elution with the predicted one and explain the differences.

**2.2. Simulations.**

*2. 2.1. Separation of the mixture*

Find the conditions to obtain acceptable resolution values by only modifying organic solvent (B) content and pH.

Explain why gradient elution is mandatory.

*2.2.2. Optimize the conditions of gradient elution*

Find gradient conditions to obtain: R>1,5 and time needed for analysis <15 min.

**Simulation Report**

Student Name 1: Date:

Student Name 2:

1. Analysis of a mixture of parabens (mix. 1)

**Question 1**: Predict the elution order of parabens and compare with the obtained chromatogram. Why dead time is evaluated by uracil?

**Question 2**: Explain how you choose the optimum wavelength.

**Question 3**: Describe how mobile phase composition in RP-HPLC affect retention and separation: `

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Acetonitrile 50% pH=7 | Acetonitrile 40% pH=7 | Acetonitrile 30% pH=7 | MeOH 40% pH=7 | Acetonitrile 40% pH=9 |
| Dead time (min) |  |  |  |  |  |
| tR butylparaben (min) |  |  |  |  |  |
| k butylparaben |  |  |  |  |  |
| Critical Resolution |  |  |  |  |  |
| Pressure (bar) |  |  |  |  |  |

**Question 4**: Describe how flow rate affect retention and separation: `

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | D=0,5mL/min | D=1mL/min | D=1,5mL/min | D=2mL/min |
| Dead time (min) |  |  |  |  |
| tR butylparaben (min) |  |  |  |  |
| k butylparaben |  |  |  |  |
| Critical Resolution |  |  |  |  |
| Pressure (bar) |  |  |  |  |

**Question 5**: Describe how stationary phase type RP-HPLC and column dimensions affect retention and separation: `

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | C18 L=150mm dp=5mm | C18  L=50mm dp=5mm | C18 L=150mm dp=3mm | C4  L=150mm dp=5mm | Phenyl  L=150mm dp=5mm |
| Dead time (min) |  |  |  |  |  |
| tR butylparaben (min) |  |  |  |  |  |
| k butylparaben |  |  |  |  |  |
| Critical Resolution |  |  |  |  |  |
| Pressure (bar) |  |  |  |  |  |
| Efficiency propyl paraben(N) |  |  |  |  |  |

**Question 6**: Propose optimum operating conditions.

*Insert Optimized chromatogram (\* paste special image)*

2. Analysis of a mixture of acids (NAIS Mix.2)

**Question 7**: Modify mobile phase composition to find isocratic conditions that separate all compounds.

**Question 8**: Predict the elution order and compare with the obtained chromatogram (with acceptable resolution values).

**Question 9**: Explain why gradient elution is preferred.

**Question 10**: Optimize gradient elution.

*Insert optimized chromatogram (\*paste special image)*